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14. ABSTRACT This is a novel study of high dose parenteral vitamin C (VitC) in a swine model of combined hemorrhagic shock and tissue trauma that simulates the course of a combat casualty by exhibiting the components of the lethal triad of acidosis, coagulopathy and hypothermia. The major goal of Year 1 of this project was to determine the effective parenteral dose of VitC. We successfully developed a reproducible traumatic injury/shock – hemorrhage model in swine. This model produced significant platelet dysfunction and a reduction in global coagulation. Histological staining (H & E) of lung showed extensive hemorrhage, septal edema, protein leak and exuberant infiltration of inflammatory cells. Significant hemorrhage and cellular damage were also evident in liver and kidney sections. Treatment with VitC (200mg/kg) was associated with a lower degree of histological tissue injury and a significantly reduced ALI score. Treatment with VitC also reduced the expression of pro-inflammatory mediators in lungs, liver and kidneys. In addition, VitC attenuated the mRNA expression of plasminogen activated inhibitor-1 and tissue factor, and increased mRNA expression of thrombomodulin. Our data suggest that intravenous VitC at 200mg/kg appears to significantly ameliorate the inflammatory status and trauma induced coagulopathy in this model.

15. SUBJECT TERMS Intravenous vitamin C, hemorrhagic shock and tissue trauma, trauma induced coagulopathy, platelet dysfunction, histological staining, pro-inflammatory mediators, coagulation markers

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Table of Contents

1. Introduction: This is a novel study of high dose parenteral vitamin C (VitC) in a swine model of combined hemorrhagic shock and tissue trauma that simulates the course of a combat casualty by exhibiting the components of the lethal triad of acidosis, coagulopathy and hypothermia. Casualty care for hemorrhagic shock and trauma involve varying degrees of inflammatory up-regulation and variable elements of coagulopathy associated with accumulating oxidative stress. This study investigated the therapeutic effects of parenteral vitamin C on inflammation and coagulation in a large animal model of hemorrhagic trauma/shock with a goal towards improving outcomes including mortality and multiple organ dysfunction.

2. **Keywords**: Intravenous vitamin C, hemorrhagic shock and tissue trauma, trauma induced coagulopathy, platelet dysfunction, histological staining, pro-inflammatory mediators, coagulation markers.

3. **Accomplishments**:

Major Goals of the Project:
During Year 1, the goal was to determine the effective parenteral dose of VitC and the accumulation of reproducible data regarding the effectiveness of the treatment. Treatment effectiveness of IV VitC were assessed based on analysis of:

a. Plasma coagulation biomarkers (tissue factor, von Willebrand factor, thrombomodulin, activated protein C, fibrinogen, and plasminogen activator inhibitor).
b. Changes in viscoelastic properties of blood (thromboelastography [ROTEM])
c. Platelet function (platelet shear modulus and platelet aggregometry, flow cytometry for surface glycoprotein expression for CD62P [P-selectin])
d. Expression of pro-inflammatory biomarkers (IL-1β, IL-4, TNFα, IL-8).

Major accomplishments under these goals:

1. Major activities:

Coordination of logistics between laboratories established: Weekly meetings between the PI, Drs. Bruce Spiess, Alpha Fowler, Donald Brophy, Charles Chalfant and Penny Reynolds culminated in the generation of a working SOP for conduct of the study. The critical aspect was defining coagulopathy in this model. Existing literature, with multitude of models has failed to provide an accurate measure of coagulopathy. Therefore a prime objective was to define coagulopathy in our model in order to provide reproducible data to assess the effectiveness of the intervention.

All required protocols approved: All Institutional and U.S. Army Medical Research and Material Command requirements (IACUC and ACURO) were approved.

Personnel responsible for study execution trained: Key technical personnel responsible for execution of the study completed training. This was a critical step to obtain reproducible data for the study.

Pilot series of trials conducted: Pilot series of trials had to be timed to avoid conflicts with other demands on the same operating suite facilities and personnel. However training of personnel and model development were completed ahead of schedule. Six animals were used during this model development phase, and blood and tissue samples processed.
Methodology (see flow chart diagram below, Figure 1)

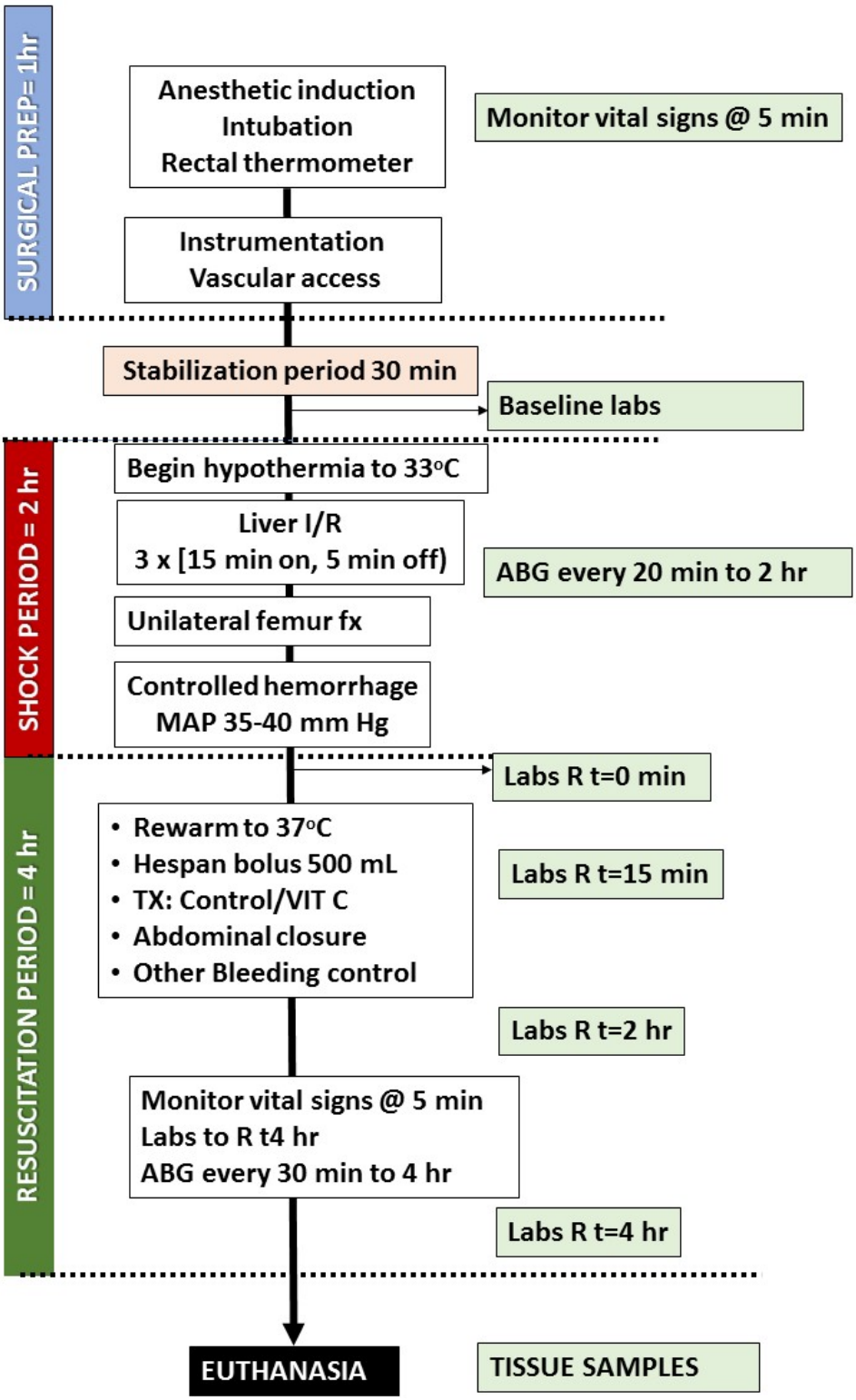
Instrumentation phase

- Animals acclimated 1-3 weeks prior to surgery; animals fasted 18 hours prior to surgery
- Animals were sedated with telazol IM, intubated, mechanically ventilated, and maintained at surgical anesthetic plane with isoflurane (balance medical air 21%); pre-surgical analgesia given IV
- Animals were instrumented for hemodynamic monitoring and allowed to stabilize for 30 min. 'Normal' acid-base status confirmed before proceeding. 'Baseline' acid-base and coagulation variables taken at this point.
- Vital signs (heart rate, SpO2, core temperature), anesthetic plane, and ventilator settings were monitored at least every 5 min for the duration of the experiment.
- Injury phase (2 hours) consisted of 4 parts:
  - Hypothermia induction: base pad cooled to induce core temperature of 33°C in approximately 30 to 40 min
  - Liver ischemia/reperfusion injury: Liver venous plexus exposed by laparotomy and I/R induced by application of ligature for 15 min followed by a release period of 5 min, repeated 3 times
  - Unilateral femur fracture with captive bolt gun (allowed to bleed freely)

- Controlled arterial hemorrhage to maintain MAP 35-40 mmHg for at least 1 hour following soft tissue/extremity/ischemia injuries

- ABG, metabolites and electrolytes measured every 20 min

Figure 1: Flow Chart for development of reproducible traumatic injury/shock- hemorrhage swine model



Resuscitation/monitoring phase

- Active rewarming to target core temperature of 36-37°C

- Abdominal closure, wound packing, other bleeding control
- Hespan bolus to 500 mL. (in definitive trial, treatment intervention i.e. saline control, or one of two doses of vitamin C will be administered at this point)
- ABG, metabolites and electrolytes measured every 30 min
- Coagulation variables taken at resuscitation time 0, 15 min, 2 hr and 4 hr.

Terminal phase
- Animal euthanized under deep anesthesia with Euthasol IV
- Tissue samples (lung, liver and kidney) obtained for histology and molecular characterization

Definitive trial conducted: Eight (8) animals from each arm of the study have been used during this phase. Hemodynamic analysis as well as molecular and functional analysis have been completed for most animals following opening of the blind.

2. Specific Objectives:

a. Determine that the animal (swine) model of traumatic hemorrhagic shock and resuscitation simulates the course of a combat casualty and exhibits the components of the lethal triad of acidosis, coagulopathy and hypothermia.
b. Assess whether resuscitation with the different doses of VitC are well tolerated by the animal following hemorrhagic trauma and shock
c. Assess whether parenteral administration of VitC attenuates circulating pro-inflammatory biomarkers.
d. Assess the dose of VitC that maximally restores hemostatic integrity
e. Assess the mechanism by which VitC normalizes platelet function in the setting of hemorrhagic trauma and shock

3. Significant results:

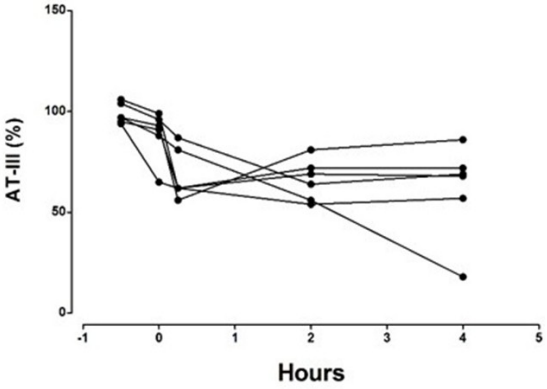


Figure 2: Changes in AT-III post-resuscitation

Model Development: We report a novel swine injury model incorporating consensus trauma-induced coagulopathy (TIC) model elements (multiple-hit injury, hypothermia, hemodilution), with liver ischemia-reperfusion (I/R) injury (to boost inflammatory response) & multivariate outcome measures. In the model developmental phase, six male Sinclair swine (36 kg) were anesthetized, intubated, mechanically ventilated, & instrumented. After 30 min physiological stabilization, a two-hour shock-injury phase (gradual forced hypothermia to 33°C, Pringle-induced liver I/R injury, femur fracture by captive bolt gun, hemorrhagic hypotension) was followed by resuscitation (500 mL Hespan bolus, active rewarming) & hemodynamic monitoring for 4 h. Coagulation measurements were obtained at baseline, end of shock, 15 min, 2 h, & 4 h. Injury resulted in significant acidosis (BE > 6), ion & liver enzyme disturbances, & leucocyte mobilization. Hespan transiently improved hemodynamics; however global coagulation dysfunction evolved throughout post-resuscitation. Although hemodilution produced a trend towards a decrease in platelet numbers, these changes did not reach statistical significance. While AT-III levels

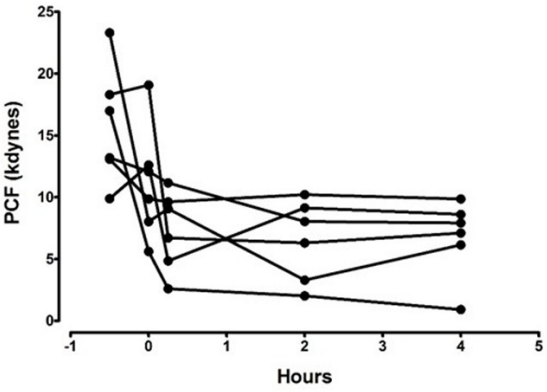


Figure 3: Changes in platelet contractile force (PCF) post-resuscitation

decreased significantly (Figure 2, 30-50%), fibrinogen levels remained relatively normal. Importantly, this model produced significant platelet dysfunction (as evidenced by decreased platelet contractile force, but no change in fibrinogen levels, Figure 3) and a reduction in global coagulation as indicated by reductions in the ROTEM Thrombodynamic Potential Index (TPI), which is a measure of global coagulation and takes into consideration both clot onset kinetics and final clot strength (Figure 4). Histological staining (H & E) of formalin perfused lung sections showed extensive hemorrhage, septal edema, protein leak and exuberant infiltration of inflammatory cells (Figure 5). Significant hemorrhage and cellular damage were also evident in liver and kidney sections. Even though the lungs and kidney were not directly subject to injury, this model induces acute coagulopathy that culminates in multiple organ injury.

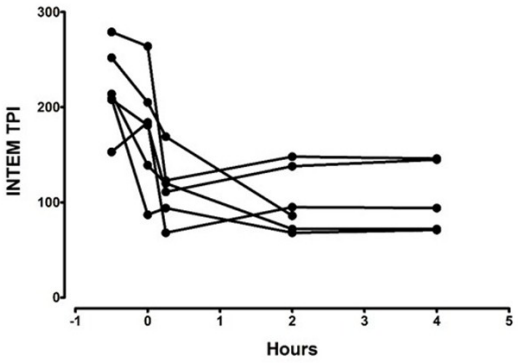


Figure 4: Changes in Thrombodynamic Potential Index (TPI) post-resuscitation

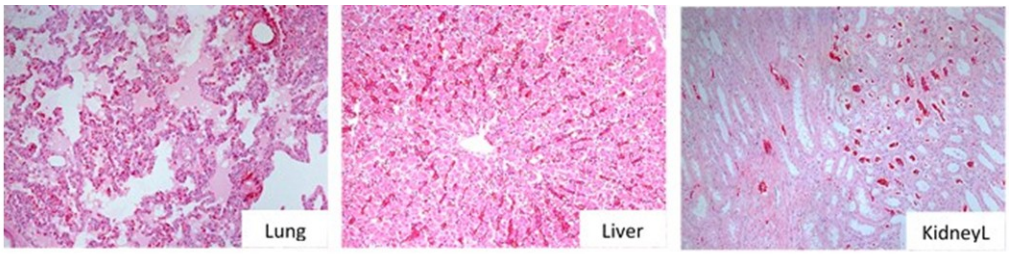


Figure 5: Histological staining (H & E) of formalin perfused lung, liver and kidney sections demonstrating significant hemorrhage and cellular injury.

Intervention trial: Treatment of swine with either dose of intravenous vitamin C was safe and well-tolerated with no aberrant hemodynamic or physiological changes evident following intervention with vitamin C. From a treatment standpoint, although there were no major changes in hemodynamic and physiological parameters, molecular changes in various organs are indicative of evolution of an anti-inflammatory and anti-coagulant phenotype following administration of intravenous vitamin C.

Plasma VitC (Figure 6): At baseline, swine in all 3 groups had a plasma VitC level of 78.5µM. Hemorrhagic shock (HS) alone induced an increase in circulating plasma VitC to 116.2µM (data not shown). This is the expected response of the animal to injury/stress. Animals in the placebo (saline) group had plasma VitC levels decline slightly (87.3µM, non-significant) over the rest of the resuscitation period (4 hours). In the Lo group, following administration of the bolus of 50mg/kg VitC, plasma levels reached a peak of 1101µM at 15 minutes post resuscitation. These levels rapidly declined over the next 4 hours to 266µM. In the Hi group, following administration of the bolus of 200mg/kg VitC, plasma levels reached a peak of 2495µM at 15 minutes post resuscitation. These levels declined over the next 4 hours to 920µM. This suggests that ongoing injury/oxidative stress results in a significant consumption/destruction of plasma VitC. Alternately, VitC is being transported from the

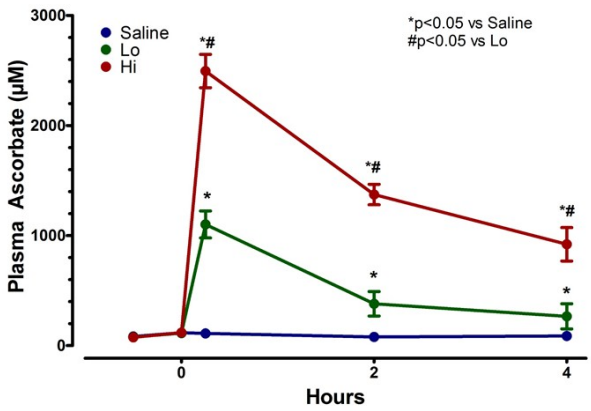


Figure 6: Changes in plasma Vitamin C levels post-resuscitation

plasma to the various tissues for maintenance of organ function or being excreted via urine.

Plasma cf-DNA levels (Figure 7): Injury produced by hemorrhagic shock and trauma did not significantly change the level of plasma cf-DNA during the resuscitation phase and with VitC treatment. A major source of cf-DNA is neutrophils undergoing NETosis. This suggests that at this early time point of resuscitation (4 hours), there is only minimal NETosis and that the contribution of NETosis to organ injury is minimal.

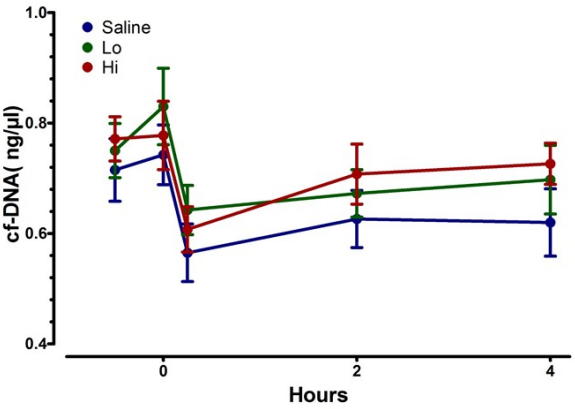


Figure 7: Changes in plasma cell free DNA (cf-DNA) levels post-resuscitation

Lung, liver and kidney mRNA expression of inflammatory (Figure 8) and pro-/anti-coagulant genes (Figure 9): The physiological environment in which trauma induced coagulopathy (TIC) arises is a complex mixture of inflammation, coagulation, and cellular dysfunction. TIC is characterized by significant pro-inflammatory events such as nuclear factor-kappa B (NFkB) activation, cytokine expression, and neutrophil infiltration. In order to assess coagulopathy and inflammation at a molecular level, we examined the mRNA expression of key mediators of coagulation and inflammation. As seen in Figure 8, treatment with VitC reduced the expression of the key pro-inflammatory mediators IL-1β, IL-8 and TNFα (not significant) in lungs, liver and kidneys. This suggest that intravenous VitC attenuated the pro-inflammatory response in multiple organs during resuscitation.

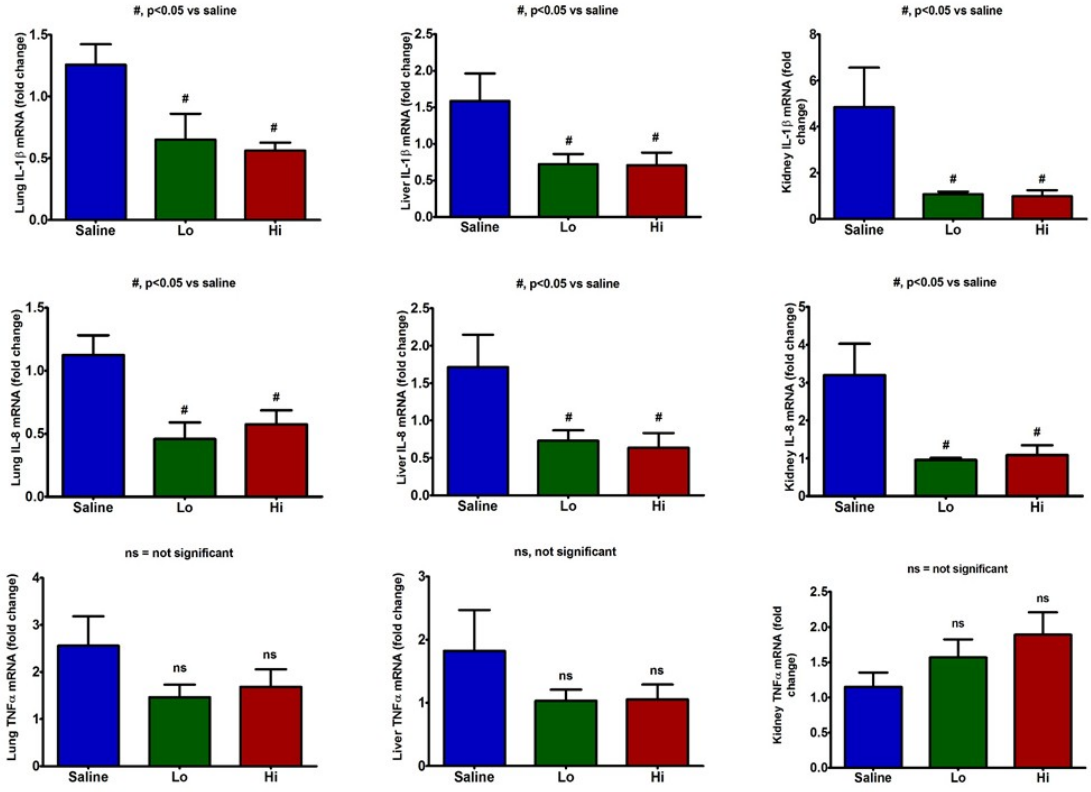


Figure 8: Relative mRNA expression of pro-inflammatory genes in lung, liver and kidney

On similar lines, treatment with VitC attenuated the lung and kidney mRNA expression of plasminogen activated inhibitor-1 (PAI-1) and tissue factor (TF) (Figure 9). PAI-1 induction in other models of HS and resuscitation was shown to be deleterious to survival. In addition to promotion of thrombosis with

subsequent tissue damage, PAI-1 was shown to play a role in damaging endothelia and hepatocytes, independent of fibrin deposition. In contrast, loss of PAI-1 protected livers from injury. TF is an NFkB driven pro-inflammatory and pro-coagulant protein. As previously demonstrated by us, it is likely that VitC attenuated TF expression by repression of the transcription factor NFkB. In contrast, VitC treatment increased the mRNA expression of thrombomodulin (TM) in lung and liver (Hi dose only). TM plays a key role in the generation of endogenous activated protein C (aPC). The anticoagulant

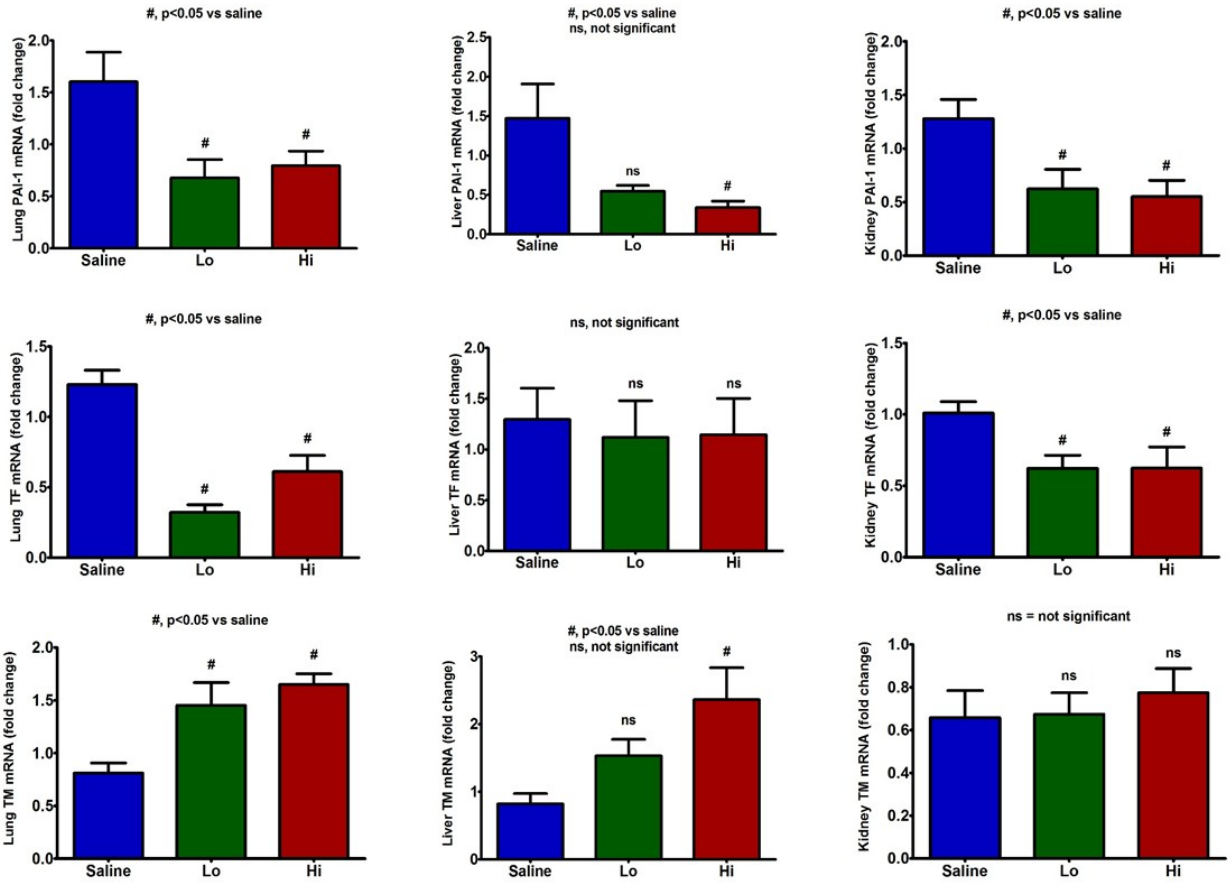
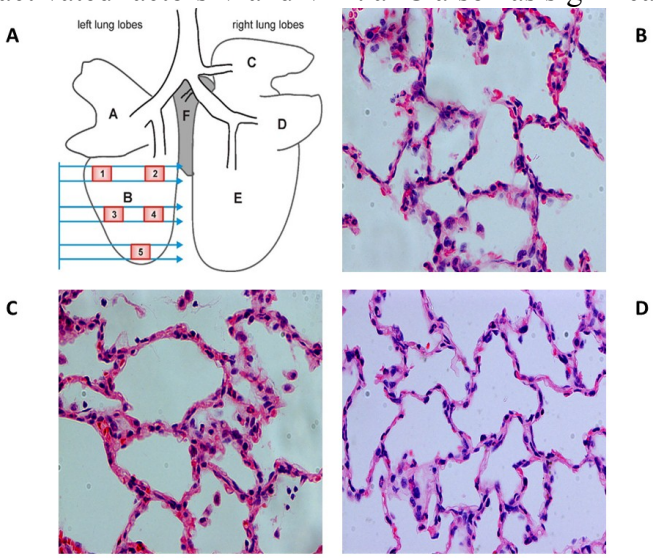


Figure 9: Relative mRNA expression of pro/anti-coagulant genes in lung, liver and kidney

properties of aPC are derived by its degradation of activated factors V and VIII. aPC also has significant anti-inflammatory properties. Investigators have shown that blocking aPC function in a murine model of TIC led to rapid mortality with massive intravascular thrombosis. Our data suggest that by inducing TM expression, VitC may promote endogenous aPC to prevent intravascular thrombosis and perhaps improve survival.

<u>Histological changes in lungs (Figures 10 and 11)</u>: After euthanasia, one lobe of liver and left kidney were removed and fixed in 10% formalin. One lobe of the left lung was removed and inflated with 10% formalin at a constant pressure of 20cm H_2O. After 7 days, tissue was removed from formalin and random sections were cut for processing and paraffin



<u>Figure 10</u>: Schematic for lung histological sections (A) and H&E staining of lung from Saline (**B**), Lo (**C**, 50mg/kg) and Hi (**D**, 200mg/kg) groups following hemorrhagic shock and resuscitation for 4 hours

embedding (Figure 10A). After embedding, 4µm sections were cut and stained using Hematoxylin and Eosin (H&E). Lung architecture was evaluated by bright-field microscopy (x10 and x40 magnification) with an Axio imager A1 microscope, Axiocam HRc camera, and AxioVision software. Ten random tissue sections from each lung were examined in each group by a blinded investigator. For each subject, a five-point scale was applied based on the recommendation of the Official American Thoracic Society Workshop Report, 0 = minimal (little) damage, 1+ = mild damage, 2+ = moderate damage, 3+ = severe damage and 4+ = maximal damage. Damage was assessed based on a) neutrophils in the alveolar or interstitial space; b) formation of hyaline membranes; c) presence of proteinaceous debris such as fibrin strands in the alveolar space; d) thickening of the alveolar wall and e) evidence of hemorrhage. Points were added up and expressed as median ± SE. As seen in Figures 10B-D, treatment with VitC, particularly at 200mg/kg was associated with a lower degree of histological tissue injury and a significantly reduced ALI score (Figure 11)

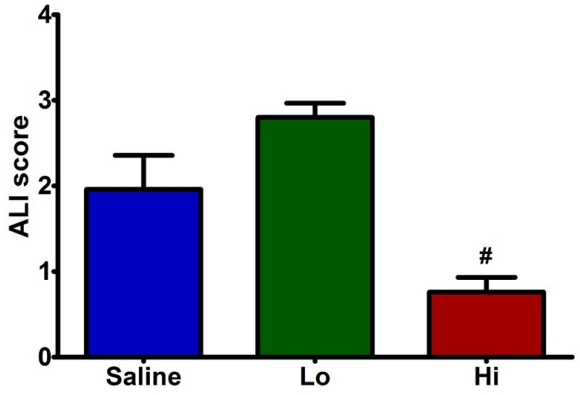


Figure 11: ALI score

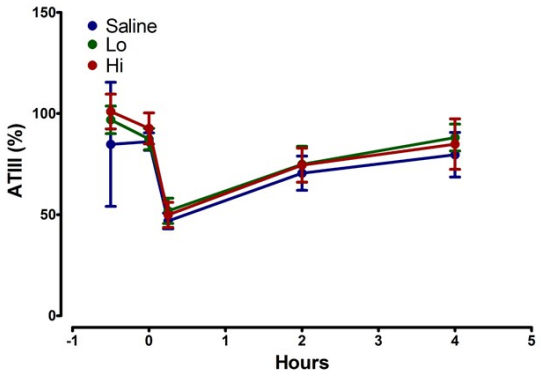


Figure 12: Changes in AT-III levels post-resuscitation

In conclusion, our stable repeatable model induced acute coagulopathy culminating in multiple organ injury. Treatment with VitC, in particular the Hi dose of 200mg/kg appears to significantly ameliorate the inflammatory status and TIC at a molecular level. Histologically also, treatment with 200mg/kg VitC improved lung morphology better than treatment with 50mg/kg. Finally, treatment with either dose of VitC was safe and well tolerated by swine with no deleterious changes in physiology or hemodynamics.

Physiological and hemostatic changes following administration of intravenous VitC: In this model of hemorrhagic shock and trauma, hemodilution produced significant platelet dysfunction (Figure 3) and alterations in various hemostasis parameters including a reduction in global coagulation as indicated by reductions in the ROTEM Thrombodynamic Potential Index (TPI), which is a measure of global coagulation and takes into consideration both clot onset kinetics and final clot strength (Figure 4). While treatment with intravenous VitC at both doses produced small trends in restoration of hemostasis parameters, none of these changes were significant at the early time point of 4 hours post resuscitation

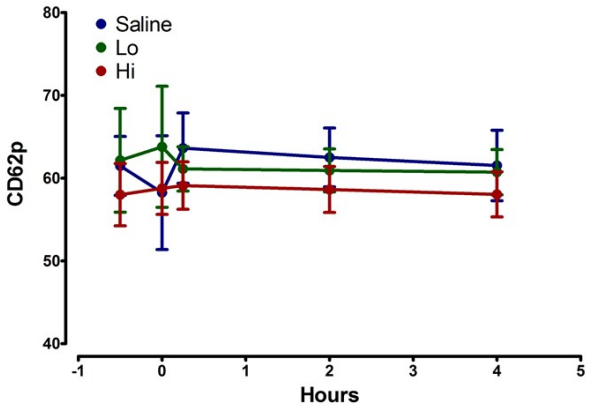


Figure 13: Changes in CD62p expression post-resuscitation

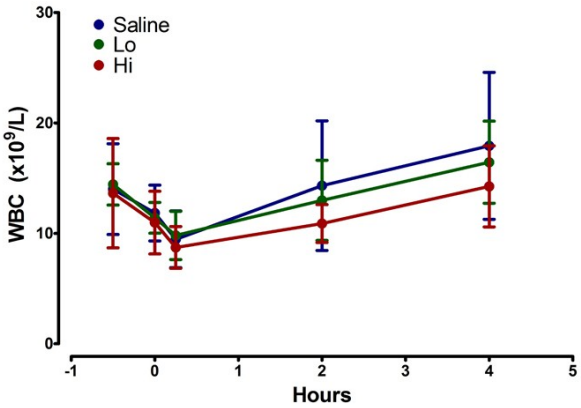


Figure 14: Changes in white blood cell count post-resuscitation

(Figures 12-17). The full list of parameters assessed in this model can be found in the Appendix section of this report.

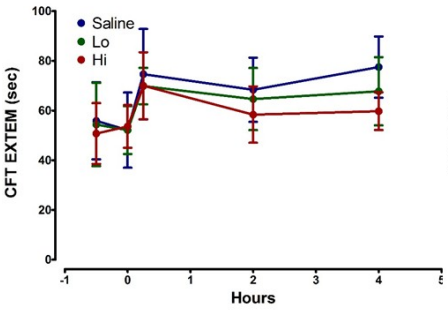


Figure 15: Changes in CFT EXTEM post-resuscitation

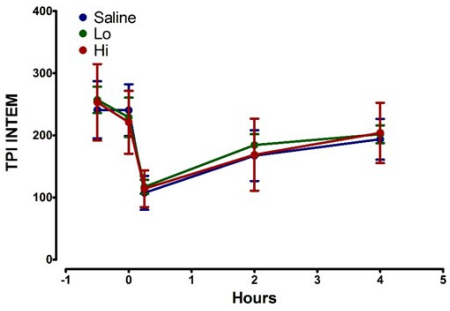


Figure 16: Changes in TPI INTEM post-resuscitation

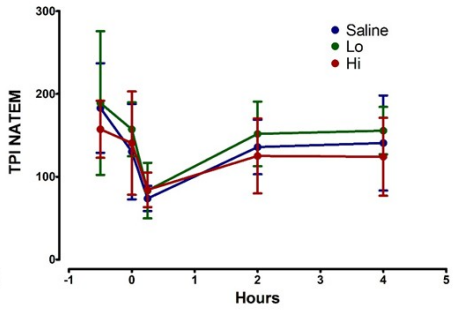


Figure 17: Changes in TPI NATEM post-resuscitation

Circulating cytokine levels following administration of intravenous VitC: We used ELISA to measure the circulating levels of the pro-inflammatory cytokines IL-1β and IL-8 in swine following injury and resuscitation and treatment with saline or Lo/Hi dose of intravenous VitC. While circulating levels trended lower with Hi dose VitC (200mg/kg), our results (Figures 18 and 19) showed no significant differences in IL-1β and IL-8 levels in swine treated with VitC. This is in contrast to the relative mRNA expression of these pro-inflammatory mediators (Figure 8). A likely explanation for this discrepancy is that the protein levels are a measure of the accumulated cytokines over the 2 hour injury period and 4 hours of resuscitation period. In contrast, the mRNA levels are a snapshot of changes occurring after 4 hours of resuscitation. These results suggest that while treatment with VitC likely altered mRNA expression levels (possibly by attenuation of the transcription factor NFκB), these changes have yet to translate to changes in the level of accumulated pro-inflammatory mediators in circulation. It is possible that a significant decline in these pro-inflammatory mediators in circulation will occur in the near future.

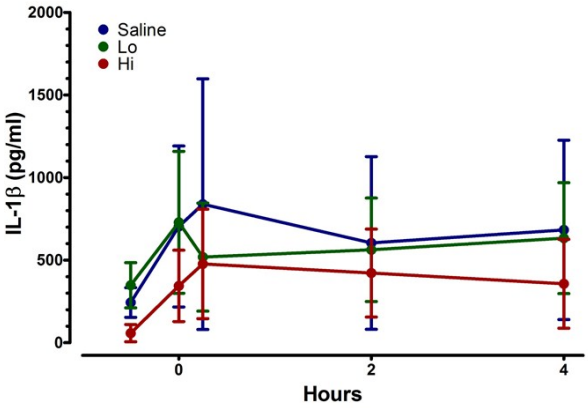


Figure 18: Changes in plasma IL-1β post-resuscitation

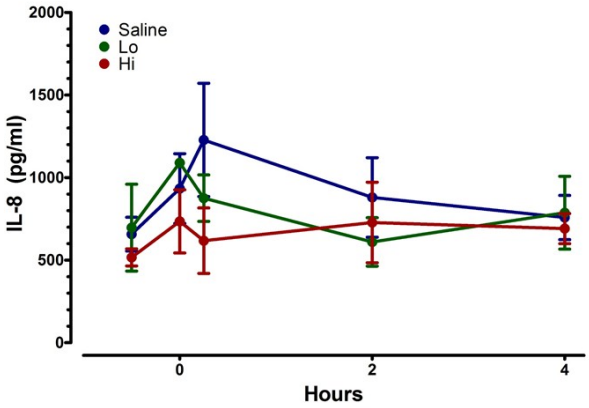


Figure 19: Changes in plasma IL-8 post-resuscitation

Other Achievements:

Alterations in Lipid Mediators during Model Development (Figures 20 and 21): Preliminary analysis of >150 analytes in plasma using UPLC mass spectrophotometry has identified a unique lipidomic signature of injury associated with hemorrhagic shock and trauma. This novel signature is characterized by suppression of d18:1 sphingosine-1-phosphate, C16 ceramide-1-phosphate, C14 ceramide, C16 ceramide, C24:1 ceramide, C24:1 sphingomyelin, C26:1 sphingomyelin and C26:1 monohexosylceramide. It remains to be determined whether treatment with VitC will improve outcomes by altering this putative injury signature.

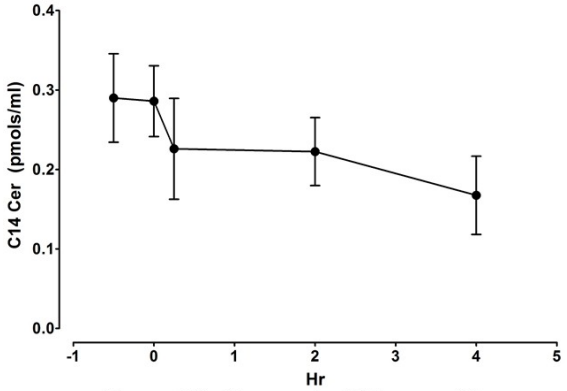


Figure 20: Changes in C14 ceramide post-resuscitation

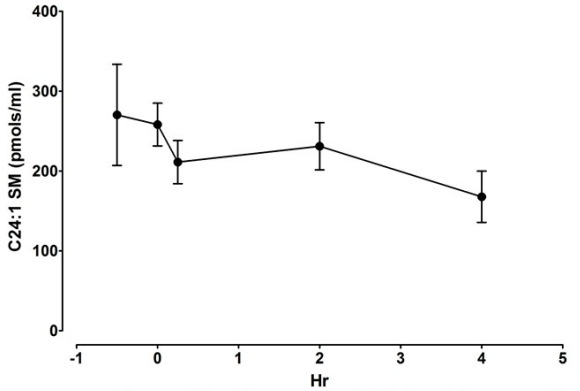


Figure 21: Changes in C24:1 sphingomyelin post-resuscitation

Longitudinal plasma proteomics study of trauma in the pig (Table 1): High-performance mass spectrometry-based proteomics was used to characterize the plasma proteomes of three control pigs collected at baseline, 0, 15, 120, and 240 minutes post-trauma. The longitudinal plasma samples were processed at the same time under identical conditions to avoid introducing bias. Raw plasma (i.e., no abundant protein removal) was reduced, alkylated, and digested with trypsin for 12 hours followed by filtration in a 10 kDa molecular weight cut-off filter. Tryptic digest samples were analyzed using a nanoflow HPLC coupled to a Q Exactive Orbitrap tandem mass spectrometer (nLC-MS/MS) in a Top-12 data dependent acquisition mode. LC-MS/MS datasets were searched in MaxQuant using the Andromeda algorithm followed by label free quantitation (LFQ).

We quantitatively monitored 308 plasma proteins over the course of the experiment for all three animals. Out of the 308 proteins, 26 were significantly different at one or more time points relative to the global mean ($p < 0.05$). Nine proteins were down-regulated relative to Baseline including Apo-CII, Apo-CIII, C1qA, C1qB, C3, ITB1, CD73, FOLR1, and ACTG1. Eight proteins were up-regulated relative to baseline including FABP1, S100-G, Apo E, BHMT, CAT, ALDOB, ALDH5, and ALDH1A1. Four proteins were initially up-regulated post-baseline but returned to baseline by 2-4 hours including CD36, FABP2, SLC9A3R1, and TUBB. The remaining five proteins (Apo M, FABP6, LYZ, PRDX6, and PEBP1) were dis-regulated relative to baseline at different time points but showed no clear trends.

A second level of interpretation focused on the presence of oxidized forms of fibrinogen. Previous studies have shown that Mox476 (oxidized Methionine 476) in human Fibrinogen-alpha chain perturbs clot formation. We searched for oxidized forms of fibrinogen in the alpha, beta, and gamma chains for all tryptic peptides detected. Four oxidized peptides were detected in all three chains including one in alpha, three in beta, and none in gamma. Importantly, the sole oxidized tryptic peptide in pig fibrinogen alpha chain aligned with the human sequence. We are now in the process of quantitatively assessing the un-oxidized/oxidized ratio as a function of time in the trauma models.

Table 1: Average LFQ data per time point

Baseline (Avg)	T = 0 min (Avg)	T = 15 min (Avg)	T = 2 hrs (Avg)	T = 4hrs (Avg)	Gene
25.59	25.52	25.39	25.07	25.00	Apo-CII
29.54	29.69	29.62	29.32	29.05	Apo-CIII
28.18	28.36	28.28	28.87	28.61	Apo E
23.57	23.98	24.84	23.45	24.62	Apo M

21.49	21.25	21.68	23.19	25.93	BHMT
21.72	21.54	22.01	23.01	24.85	CAT
24.41	24.14	23.66	23.76	23.50	C1qA
24.83	24.54	23.44	23.95	21.38	C1qB
32.71	32.69	32.66	32.55	32.39	C3
20.70	22.03	21.34	21.12	19.18	CD36
21.93	23.75	23.20	21.84	20.31	FABP2
21.55	26.80	27.29	25.58	25.87	FABP1
20.95	24.25	24.27	20.11	22.30	FABP6
20.72	20.70	20.87	20.58	19.87	ITB1
21.93	20.70	22.29	21.34	20.60	LYZ
20.59	21.04	21.07	21.24	19.80	SLC9A3R1
22.03	20.59	22.34	22.37	21.35	PRDX6
21.23	25.16	25.16	24.36	23.55	S100-G
22.30	20.37	20.88	21.40	20.78	FOLR1
21.42	21.75	22.96	21.38	20.19	TUBB
21.49	20.62	20.82	20.59	20.44	ACTG1
21.47	19.47	21.26	20.48	22.16	PEBP1
21.48	21.40	20.91	21.86	23.84	ALDOB
22.03	22.42	21.72	21.65	25.26	ADH5
22.01	21.25	21.60	21.06	23.22	ALDH1A1
21.75	22.38	22.38	21.94	20.70	CDC73

Opportunities for training and professional development:

While this study was not designed to specifically provide training and professional development, it allowed the surgery technicians and the histopathologist to advance their skills. In particular, the beneficiaries of this were: Christopher Sweeney, Jacquelyn McCarter, Daniela Farkas, Paul Middleton and Matthew Ellenberg.
In addition, Dr. Penny S. Reynolds, Co-Investigator on the study, presented the model development aspect of the study with an abstract titled “Development Of A Novel Swine Model of Trauma-Induced Coagulopathy”. This was accepted as a poster presentation at the 2016 Military Health System Research Symposium (MHSRS) in Orlando/Kissimmee, FL.

Dissemination of results:

The model development aspect of the study was presented with an abstract titled "Development Of A Novel Swine Model of Trauma-Induced Coagulopathy". This was accepted as a poster presentation at the 2016 Military Health System Research Symposium (MHSRS) in Orlando/Kissimmee, FL.

Future Plans for next reporting period:

- Assess the dose of VitC that maximally restores hemostatic integrity: In keeping with the proposed SOW, in the first quarter of Year 2, we will modify the dose of IV VitC. In particular, we will add a second dose of treatment (saline, Lo [50mg/kg] or Hi [200mg/kg]) after 2 hours of resuscitation. The rationale behind this addition is based on data obtained in Year 1. In the Lo group, following administration of the bolus of 50mg/kg VitC, plasma levels reached a peak of 1101μM at 15 minutes post resuscitation. These levels rapidly declined over the next 4 hours to 266μM. In the Hi group, following administration of the bolus of 200mg/kg VitC, plasma levels reached a peak of 2495μM at 15 minutes post resuscitation. These levels declined over the next 4 hours to 920μM. This suggests that ongoing injury/oxidative stress results in a significant consumption/destruction of plasma VitC. We anticipate this to be completed by the end of the second Quarter of Year 2 (3 groups, one control, 2 doses of intravenous Vitamin C, N=6 per group)
- Complete data analysis on remaining swine from Year 1.
- Initiate novel lipidomic and mass spectrophotometric analysis on the model development group.

4. **Impact**:

Impact on the development of the principal discipline of the project:
Short-Term Impact: Immediate (2 to 5 years) applications of this project will be research-related in 4 focal areas of impact. Traditional avenues of academic sector knowledge production will include dissemination of research findings by publication in peer-reviewed journals that cover both clinical and basic research, at conferences, and in research reports. Basic data will provide comprehensive documentation of the coagulation process though the short-term shock/injury/resuscitation cycle, and will enable investigation of the potential of VitC to reduce inflammation and coagulopathy secondary to massive system trauma. The translatability potential of this research is very great. Because our team measures clinically-relevant metrics – global coagulation dysfunction, inflammation, hemodynamics – our findings will provide a rationale for proceeding to human clinical trials in the near future. Finally the methodological contribution to this area of research is novel in that it represents an extension of process quality control improvement and product optimization strategies to preclinical research to enable rapid convergence to an optimal solution. Application of these techniques, together with traditional methods of reducing bias, such as randomization and blinding, will ensure high-quality data, and be a model for other investigations of this type.

Long-Term Impact: The vision for this research is to expedite the development, clinical trial testing, and FDA certification of this potentially life-saving therapeutic, so as to enable deployment in the far-forward arena in the immediate near future. Our expectation is that intravenous preparation of VitC will prove to be a safe and effective field adjunct to current standard-of-care resuscitation products, be rapidly integrated into prehospital care protocols, and most importantly, act to minimize long-term sequelae of sepsis and MOF in surviving combat casualties.

Military Benefit: Rapid, effective resuscitation is essential for treating combat injuries with active bleeding and hypovolemia. In the far-forward arena, treatment of the wounded war-fighter is most challenging in the earliest time period following injury. Injuries are frequently multimodal (blast, penetrating, blunt), and at significant risk for infection. The amount of resuscitation fluid available in the field may be limited. Provision of large amounts of fluid is not often practical under fire or in austere environments, and optimizing allocation of scarce fluid resources according to triage criteria means that many wounded receive inadequate or no supportive fluid therapy. Logistic problems of applying stabilizing treatment in combat conditions are compounded by delayed evacuation from the hot zone. Unfortunately, this is when specific treatments need to be applied to have the greatest therapeutic benefit. Furthermore, current fluid resuscitation strategies do not reduce risk for late-term sepsis and MOF. Based on our previous calculations, low volumes of unaugmented colloids or crystalloids are unlikely to reverse or prevent severe cell and organ damage; these fluids may actually induce inflammation and coagulopathy in casualties with moderate to severe hemorrhage and polytrauma. VitC is significantly depleted in critical injury and illness; parenteral VitC administration to critically ill sepsis patients has been demonstrated to reduce the severity of end-organ damage and mortality. If the military remains committed to low-volume battlefield resuscitation, our approach – combining an approved antioxidant (VitC) with an approved colloid or crystalloid – is a realistic, cheap, and effective strategy to prevent additional mortality and morbidity, with a high probability of rapid (3-5 years) incorporation into standard of care protocols. Supplementation of resuscitation fluids already in common use would require little revision of medic skills. Instead, casualties could receive one or two IV product applications close to point of injury and/or enroute to definitive care. Product could be reconstituted with whatever approved fluid was on hand (crystalloid, colloid) and easily infused via peripheral vascular access or intra-osseous device, as established per protocol. This fluid resuscitation strategy would meet the medic's objectives of keeping the casualty alive in the short term, with the added benefit of minimizing cell damage that occurs during these early stages of traumatic injury, and

which contributes to sepsis and end-organ damage in the longer term. Small volume high-dose formulations would meet logistic constraints on weight and volume.

Impact on other disciplines:

If successful, the impact of this therapeutic will be broadly applicable to a variety of critically-ill civilian surgical and medical patient populations, also at high risk for systemic inflammation and development of MOF. Current sepsis treatment involves prolonged hospital stays in the intensive care unit at very high cost (over $20 billion in 2011 alone, or over $55 million per day). Sepsis patients are hospitalized longer, more likely to be discharged to facilities other than home, and suffer high rates of readmission, resulting in additional costs of over $2 billion per year. Therefore if this product shows demonstrable reduction or prevention of runaway inflammation and TIC, early-stage intervention could be expected to greatly reduce these costs. In addition, our investigations of the cellular and molecular underpinnings of inflammatory and coagulation responses could serve as the basis for future design of "smarter", more targeted, resuscitation strategies and therapeutics. The long-term impact of successful trialing of this product will therefore be service-related because of: improvements in public health through better treatment; potentially enormous cost savings and cost containment through reductions in hospital stays and intensive care; changes in evidence-based practice (through provision of evidence affecting treatment decisions, and clinical practice), and quality of care (assuming the efficacy of this health intervention).

Currently available pharmacologic agents: Treatment for sepsis is mainly supportive, involving large amounts of fluid, antibiotics, vasopressors, corticosteroids, conservative mechanical ventilation and immunomodulatory drugs. However the major challenge of treatment is diagnosis; early recognition of sepsis is difficult, and treatment may be delayed until the condition is far advanced. In lieu of focusing on early detection, treatment with VitC close to point of injury may be a simple, cost-effective, and clinically effective alternative.

Impact on technology transfer:

Nothing to report

Impact on society beyond science and technology:

Public Purpose: Countless medical interventions derived from military medicine have been incorporated into civilian prehospital medicine and trauma surgery. Our proposed VitC therapeutic could be readily applied to civilian patient populations characterized by compromised blood flow to organs, and thus at significant risk of developing sepsis and MOF. More than 1 million patients in the US develop sepsis each year, and over 50% die; health care costs may exceed $20B per year. Vulnerable populations include patients with hemorrhagic shock (resulting from blunt trauma, penetrating trauma, surgical mishaps, and gastrointestinal bleeds), hemodiluted surgical patients, and patients with medical emergencies, such as stroke or cardiac arrest. The prevention or reduction of sepsis and organ failure is thus of significant public health interest. Finally there is the potential for a large societal impact, if this research can prove to contribute to improvements in the care and management of the critically ill and injured by reducing morbidity and mortality, and through economic benefits resulting from direct cost savings to health care systems, and through reduction in lost productivity of a relatively young patient cohort.

5. **Changes/Problems**:

Changes in approach and reasons for change:

Our approach has remained essentially identical to that proposed in the SOW. One additional step incorporated was the induction of significant liver injury by ischemia/reperfusion (using a modified Pringle technique). This change was necessitated due to the ability of the animal to compensate and adapt to the injury. Addition of ischemia-reperfusion of the liver using the modified Pringle technique initiated an inflammatory cascade and induced coagulopathy that better simulated combat injury.

Actual or anticipated problems or delays and actions or plans to resolve them:

Logistic challenges: Scheduling conflicts with other DoD funded studies requiring the same room, equipment, and technical personnel.
Solution: Find the best compromise and flexible workarounds for shared resources.

Technical challenges: This is a highly complex protocol with the potential for numerous human errors.
Solution: Regular pre- and post-experiment briefings, protocol checklists (as is the new norm for human surgery) and protocol flowcharts. These are regularly updated with input from all personnel, and posted for easy reference.

Personnel challenges: Dr. Bruce Speiss, Co-PI, and Dr. Penny Reynolds, Co-I joined the University of Florida, Gainesville in mid-April and end of June, respectively
Solution: The rest of the surgical and study team were unchanged as described in the SOW: they took up additional responsibilities that were previously handled by Dr. Reynolds and Dr. Spiess. Jacquelyn McCarter and Christopher Sweeney were designated for 100% effort on this study. They covered most of the additional responsibilities previously handled by Dr. Reynolds.
Two additional part time personnel (hourly) were hired starting July 1. Since their hire, Matthew Ellenberg and Paul Middleton have been actively assisting the surgery team with intubations, animal monitoring, record keeping and tissue processing.

Changes that had a significant impact on expenditures:

The study remained financially healthy throughout Year 1 and ended up below projected budget by about $50,000. These funds will be carried over to Year 2 for performance of additional assays, purchasing supplies, for travel to meetings and for publication charges.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents:

No significant changes were made in the use or care of vertebrate animals, biohazards or select agents. ACURO approval was obtained on 03 June 2015. The protocol was approved by VCU-IACUC on 15 April 2015. A minor modification was made on 14 March 2016, which was approved by VCU-IACUC:

- Addition of qualified personnel to roster
- Changes in pre-surgical sedation/induction drug (to tiletamine/zolezepam 5mg/kg from ketamine/xylazine)
- Changes in resuscitation fluid from HEXTEND to HESPAN (due to availability issues)
- Change in swine strain from Hanford to Sinclair minipigs (due to availability issues)

6. **Products:**

Publications, conference papers, and presentations:

Reynolds PS, Natarajan R, Brophy D, Martin E, McCarter J, Middleton P, Sweeney C, Thummala S, Spiess B. Development of A Novel Swine Model of Trauma-Induced Coagulopathy. 2016 Military Health System Research Symposium (MHSRS), Orlando/Kissimmee, FL

Website(s) or other Internet site(s):

Nothing to report

Technologies or techniques:

This study employs an operationally-standardized preclinical swine model of traumatic injury and hemorrhage developed by the PI, and extensively validated in pilot and initial definitive testing by our team. It incorporates all consensus elements of an animal coagulopathy model: significant 'multiple hit' tissue injury, a combination of controlled and uncontrolled mild to moderate hemorrhage, prolonged hypotension, hemodilution (simulating clinical resuscitation practice), hypothermia, acidosis, measurement of inflammatory markers, and assessment of anticoagulant and fibrinolytic pathways. In addition, our model applies iatrogenic injury and resuscitation procedures in the clinically relevant and appropriate chronological order (e.g. hemodilution occurs after injury). The shock/injury phase involves both hemorrhage and isolated traumatic insults, including unilateral femur fracture and solid organ injury. A novel feature of our model is the application of a standardized Pringle maneuver to impose an intermittent liver ischemia/reperfusion (I/R) injury to induce inflammation and coagulopathy. The combined trauma-hemorrhage model optimizes operational characteristics such as clinical relevance, standardization, reliability, and reproducibility.

Inventions, patent applications, and/or licenses:

Nothing to report

Other Products:

Nothing to report

7. **Participants & Other Collaborating Organizations:**

What individuals have worked on the project?

Name	Ramesh Natarajan
Project Role	Principal Investigator
Nearest person month worked	5
Contribution to project	Getting protocols approved; Training required personnel; Development of working SOP; Development of reproducible traumatic injury/shock-hemorrhage swine model; Performed definitive Trial for intervention with intravenous vitamin C; Analyzed data; Prepared reports; Singularly responsible for conduct of project.

Name	Bruce Spiess
Project Role	Co-Principal Investigator
Nearest person month worked	0.03
Contribution to project	Guided the evolution of the model and assisted the PI with data interpretation and analysis of treatment outcomes.

Name	Penny Reynolds
Project Role	Co-Investigator
Nearest person month worked	4.5
Contribution to project	Getting protocols approved; Training required personnel; Development of working SOP; Development of reproducible traumatic injury/shock-hemorrhage swine model; Assisted the PI to perform initial portion of definitive Trial for intervention with intravenous vitamin C.

Name	Alpha Fowler
Project Role	Co-Investigator
Nearest person month worked	0.06
Contribution to project	Guided the evolution of the model and assisted the PI with data interpretation and analysis of treatment outcomes.

Name	Bernard J Fisher
Project Role	Co-Investigator
Nearest person month worked	1.35
Contribution to project	Measured plasma vitamin C levels and cell free DNA levels; Performed cytokine and molecular analysis for expression of genes related to inflammation and trauma induced coagulopathy; Assisted the surgical team with sample preparation and collection.

Name	Donald Brophy
Project Role	Co-Investigator
Nearest person month worked	0.12
Contribution to project	Assessed coagulation changes and platelet function in the model and following treatment.

Name	Erika Martin
Project Role	Co-Investigator
Nearest person month worked	1.2

Contribution to project	Performed assays for all coagulant-anticoagulant and fibrinolytic pathways; Assessed coagulation changes and platelet function in the model and following treatment.

Name	Jacquelyn McCarter
Project Role	Veterinary Laboratory Specialist
Nearest person month worked	7.5
Contribution to project	Getting protocols approved; Training required personnel; Development of working SOP; Performing surgeries for development of reproducible traumatic injury/shock- hemorrhage swine model.

Name	Christopher Sweeney
Project Role	Veterinary Laboratory Specialist
Nearest person month worked	7.5
Contribution to project	Getting protocols approved; Training required personnel; Development of working SOP; Performing surgeries for development of reproducible traumatic injury/shock- hemorrhage swine model.

Name	Daniela Farkas
Project Role	Histology Specialist
Nearest person month worked	0.6
Contribution to project	Histological assessment of lung, liver and kidney injury.

Name	Paul Middleton

Project Role	Veterinary Laboratory Specialist
Nearest person month worked	6
Contribution to project	Development of working SOP; Performing surgeries for development of reproducible traumatic injury/shock- hemorrhage swine model; Responsible for intubation of swine and maintaining the ventilator

Name	Matthew Ellenberg
Project Role	Veterinary Laboratory Specialist
Nearest person month worked	1.5
Contribution to project	Development of working SOP; Performing surgeries for development of reproducible traumatic injury/shock- hemorrhage swine model; Responsible for record keeping and assisting with all aspects of surgery.

Name	Dayanjan Wijesinghe
Project Role	Assistant Professor
Nearest person month worked	0.6
Contribution to project	Performed Lipidomics analysis on plasma from swine used for injury development.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Nothing to report

8. **Special Reporting Requirements**:

Collaborative Awards:

N/A

Quad Charts:

Please see after Appendix.

9. **Appendices**:

List of other hemostasis and physiological parameters investigated:

CBC Parameters:
WBC X 109/L
RBC X 1012/L
HGB g/dL
HCT %
MCV fl
MCH pg
MCHC g/dL
RDWC %
PLT x 109/L
PCT %
MPV fl
PDWC %
LY%
MON%
NE%

Coagulation Parameters:
Fib (mg/dL)
vWF:Ag (%)
aPC (%)
ATIII (%)
COLL/ADP PFA (sec)
COLL/EPI PFA (sec)
FOT (min)
PCF (kdynes)
CEM (kd/cm2)
CT NATEM (sec)
CFT NATEM (sec)
Angle NATEM (degree)
MCF NATEM (mm)
NATEM TPI
CT INTEM (sec)
CFT INTEM (sec)
Angle INTEM (degree)
MCF INTEM (mm)
INTEM TPI
CT EXTEM (sec)
CFT EXTEM (sec)
Angle EXTEM (degree)
MCF EXTEM (mm)
EXTEM TPI
Flow Cytometry for CD62p + ADP

Physiological Parameters (Bio-Pac):
MAP, PAP, CVC, CCO